PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Issam RAAD, Hend A. HANNA, and Nabeel NABULSI	Group Art Unit: 1744
Serial No.: 10/044,842	Examiner: Jastrzab, Krisanne Marie
Filed: January 11, 2002	Atty. Dkt. No.: UTSC:669US
For: NOVEL ANTISEPTIC DERIVATIVES WITH BROAD SPECTRUM ANTIMICROBIAL ACTIVITY FOR THE IMPREGNATION OF SURFACES	

SECOND DECLARATION OF DR. ISSAM RAAD UNDER 37 C.F.R. § 1.132

I, Dr. Issam Raad, hereby declare as follows:

1. I am one of the inventors of the above-referenced patent application. I am a citizen of the U.S., currently residing at 4207 Clearwater Ct., Missouri City, TX, 77459.

2. The Example section of the above-referenced patent application provides strong evidence of synergy of gentian violet (GV) and chlorhexidine (CHX) as an antiseptic/disinfectant. I use the term "synergy" to refer a combined action of basic reagent and dye that is greater than the expected action of the basic reagent and dye separately. Table 2 and Table 3 on page 20 of the referenced patent application show zones of inhibition (ZOI) produced by coated endotracheal PVC tubes (using DCM or MeOh). As set forth in the application on page 20, lines 16-19, "endotracheal PVC tubes impregnated with Gendine (GN) are far more effective against all

organisms when compared with those impregnated with CHX, and more effective than PVC tubes impregnated with GV against *Pseudomonas aeruginosa*."

3. Table 4 on page 21 of the referenced application shows ZOI produced by coated silicone catheters. Page 21, lines 10-12 states that "data in Table 4 shows how silicone catheters impregnated with GN are more effective in inhibiting *MRSA, PS* and *C. parapsilosis* than catheters impregnated with either GV or CHX."

4. Table 5, on page 21 of the present application, shows ZOI produced by coated polyurethane catheters (PU). Page 21, lines 24-27 states that "PU catheters impregnated with GN are more effective than PU catheter impregnated with GV in inhibiting *Pseudomonas aeruginosa*, and more effective than PU catheters impregnated with CHX against all three organisms, *MRSA, PS* and *C. parapsilosis*."

5. Table 6, on page 22 of the present application, shows ZOI produced by coated silk sutures. Page 21, lines 10-12 provides that "silk sutures coated or impregnated with GN are significantly more effective in inhibiting MRSA, PS and *C. parapsilosis* than sutures coated with either GV or CHX."

6. Similarly, Tables 7-10 on pages 24-25 show similar synergy against various bacterial and fungal organisms, when GV was combined with other basic reagents on the surfaces of medical devices.

7. Furthermore, as discussed in my first declaration (filed with the response to the Office Action dated January 11, 2006), I have provided additional evidence demonstrating that the combination of a basic reagent and a dye has antiseptic ability as a mouthwash, coating of a glove, or coating of a catheter than is more than additive compared to either dye alone or basic reagent alone (see Exhibit 1 of my first declaration).

8. In addition, attached as Exhibit 1 of this declaration is a summary of data from my laboratory that further demonstrates a high level of synergy of the combination of a basic reagent and a dye in antiseptic ability.

9. The most serious forms of catheter related bloodstream infections are those caused by fungi, particularly *Candida albicans*. This is the infection with the highest mortality rate – around 40%. We have found that gendine (GV and CHX) mixed in a specific molar ratio to coat catheters and devices provides unexpectedly superior synergy against *Candida albicans*. The strain used in the studies summarized in Exhibit 1 was obtained from a patient who suffered from catheter-related fungemia/candidemia caused by *Candida albicans* (strain 009-3072). In the first part of the study summarized in Exhibit 1, we calculated a minimal inhibitor concentration (MIC) and minimal fungicidal concentration (MBC) for each of the components, GV and CHX. The MIC/MBC was 0.5 microgram per mL for the GV and 16 microgram per mL for CHX.

10. When we tested for synergy of the combination of CHX and GV over a range of 1:1 to 100:1, and we obtained the results described on page 2 of Exhibit 1. Boxes that are shaded had a complete kill of the *Candida albicans* at the respective concentrations of the components that are

lower that the MIC and MBC of CHX alone and GV alone. The best synergistic data was obtained at a ratio of CHX:GV of 1:1 and 10:1, with a plateauing effect at 25:1 and thereafter. In other words, there is synergy obtained at 50:1 and 100:1 but it is not appreciably different from 25:1.

11. These results clearly establish that the claimed methods using a combination of a dye and basic reagent are surprisingly and unexpectedly superior compared to methods of disinfecting using dye alone or basic reagent alone.

12. Further, my group has published a study (Bahna *et al.*, Oral Oncol. 2007 Feb.; 43(2):159-64; Exhibit 2) that demonstrates that mouthwash compositions that have a ratio of dye:basic reagent of 10:1-66:1 demonstrated synergistic antimicrobial efficacy against free-floating and biofilm forms of *Candida albicans*.

13. I hereby declare that all statements made by my own knowledge are true and all statements made on information and belief are believed to be true and further that statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment under § 100 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereon.

Date 4/20/07

IRaad

Issam Raad

EXHIBIT 1

Efficacy and Potency of Gendine and its components against Candida albicans.

Objective:

1. To determine potency (MIC, MBC) for the components of Gendine (Gentian Violet and Chlorhexine) as well as optimal concentration of both components mixed together to form Gendine.
2. To determine potency (MIC, MBC) for the molar ratios of Gendine (Chlorhexidine:Gentian Violet).

Materials and Methods:

Potency of Gendine, Gentian Violet and Chlorhexidine –
Using standard techniques to determine MIC and MBC, gentian violet and chlorhexidine were tested independently against *Candida albicans* (009-3072).

Potency of Molar Ratios of Gendine
Using standard techniques to determine MIC and MBC, molar ratios (25:1, 10:1, and 1:1) of the combination of gentian violet and chlorhexidine were tested against *Candida albicans* (009-3072).

Results:

Potency of Gendine, Gentian Violet and Chlorhexidine –

		CA 009-3072
Gentian Violet	MIC (µg/mL)	0.5
	MBC (µg/mL)	0.5
Chlorhexidine	MIC (µg/mL)	16
	MBC (µg/mL)	16

Potency of Molar Ratios of Gendine –

Red = Kill, Black = Growth

Possible synergistic effect of Gendine is shown highlighted in yellow (kill at concentrations below both individual MIC)

All Ratios shown are CHX:GV

Experiment - November 3, 2006

C.albicans 009-3072

	+ control	1	2	3	4	5	6	7	8
100:1	MHB	0.192:0.0016	0.384:0.0032	0.767:0.0064	1.534:0.013	3.069:0.026	6.14:0.051		
50:1	MHB	0.192:0.0032	0.384:0.0064	0.767:0.013	1.534:0.026	3.069:0.051	6.14:0.103		
25:1	MHB	0.192:0.0064	0.384:0.013	0.767:0.026	1.534:0.051	3.069:0.103	6.14:0.205		
10:1	MHB	0.157:0.0064	0.315:0.013	0.632:0.026	1.264:0.103	2.527:0.205	5.055:0.41		
1:1	MHB	0.063:0.051	0.126:0.103	0.253:0.205	0.505:0.41	1.01:0.82	2.02:1.64	4.04:3.28	8.08:6.56

CHX:GV	MIC
100:1	6.14 : 0.051
50:1	6.14 : 0.103
25:1	6.14 : 0.2.05
10:1	2.527 : 0.205
1:1	0.253 : 0.205

CHX MIC 16 **GV MIC** 0.5

EXHIBIT 2

Oral Oncology (2007) 43, 159–164

available at www.sciencedirect.com

ScienceDirect

ORAL ONCOLOGY

ELSEVIER

journal homepage: http://intl.elsevierhealth.com/journals/oron/

Antiseptic effect of a novel alcohol-free mouthwash: A convenient prophylactic alternative for high-risk patients

Paul Bahna [a], Hend A. Hanna [a,*], Tanya Dvorak [a], Ara Vaporciyan [b], Mark Chambers [c], Issam Raad [a]

[a] *Department of Infectious Diseases, Infection Control and Employee Health, The University of Texas, M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Unit 402, Houston, TX 77030, United States*
[b] *Department of Thoracic and Cardiovascular Surgery, The University of Texas, M.D. Anderson Cancer Center, TX, United States*
[c] *Department of Dental Oncology, The University of Texas, M.D. Anderson Cancer Center, TX, United States*

Received 21 November 2005; received in revised form 2 February 2006; accepted 3 February 2006
Available online 22 June 2006

KEYWORDS
Brilliant green;
Biofilm;
Antiseptic mouthwash

Summary We developed an efficacious and non-irritant mouthwash that is alcohol-free and that has a low concentration of chlorhexidine, in order to be used for preventing oral cavity infections in immunocompromised and cancer patients. The novel mouthwash solution was tested for its antimicrobial efficacy against both free floating (planktonic) and the biofilm forms of *Candida albicans*. The solution was also tested against *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and methicillin-resistant *Staphylococcus aureus* (MRSA), using a modification of a previously published method. The activity of the novel mouthwash was also compared with that of three commercially available mouthwashes. The experimental mouthwash showed efficacy against *C. albicans*, both in free-floating form and in biofilm. With higher concentration of chlorhexidine, the solution was also efficacious in inhibiting the growth of *K. pneumoniae*, *P. aeruginosa*, and MRSA. The antiseptic activity of the alcohol-free mouthwash against other bacterial organisms and *C. albicans* was comparable to other commercially available alcohol-based mouthwash solutions. A novel alcohol-free mouthwash solution, that has low concentration of chlorhexidine, showed antiseptic effect against planktonic and biofilm forms of *C. albicans* and against *K. pneumoniae*, *P. aeruginosa*, and MRSA.

* Corresponding author. Tel.: +1 713 792 7943; fax: +1 713 792 8233.
E-mail address: hhanna@mdanderson.org (H.A. Hanna).

1368-8375/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.oraloncology.2006.02.002

Introduction

In critically ill and immunocompromised patients, the oral cavity is a common colonization site for numerous multidrug resistant bacterial and fungal microorganisms that can cause infections. Oral candidiasis is highly prevalent among immunocompromised patients and patients with dentures.[1–3]

There has been an increase in the number of immunocompromised patients, over the past few decades, caused in part by the rise in the numbers of bone marrow and solid organ transplantations, the increasing number of patients needing critical care, and the aggressive use of chemotherapy and radiation therapy.[4] Oral mucositis is recognized as a common complication of radiation therapy in patients with head and neck carcinoma, and currently, its treatment is essentially palliative.[5] Additionally, *Candida*-associated stomatitis is also a recognized complication in elderly denture users, especially when denture hygiene is lacking.[6] Epstein et. al. found out that oropharyngeal colonization by *Candida* species was common in recipients of hematopoietic cell transplants, despite systemic and topical antifungal prophylaxis.[7] They also showed that patients who underwent total-body irradiation and who had evidence of *Candida* colonization were at higher risk of death after their transplant than those who were *Candida*-negative ($P < .001$).[7]

Furthermore, fungal microorganisms such as *Candida* species are among the most common microorganisms causing nosocomial bloodstream infections in the United States. Although *Candida* species are part of the normal mouth flora in 25–50% of healthy individuals, often referred to as asymptomatic colonization,[8] this rate tends to be higher in patients with debilitating diseases such as HIV patients[9], diabetic patients,[10] and patients with cancer,[11] where *Candida* are more prone to cause symptomatic disease rather than mere asymptomatic colonization. Within five years of seroconversion, up to 26% of HIV positive patients develop oral candidiasis, which is seen also in 12–100% of cancer patients undergoing chemotherapy, according to a published analysis of 15 studies, and in 76% of patients undergoing bone marrow transplant, and in up to 77% of carefully followed-up asthmatics using inhaled corticosteroids.[12–15]

Bacterial microorganisms can either migrate or be aspirated from the oropharynx throughout the respiratory system, and cause pneumonia. In addition, the microorganisms can form biofilm along the surface of an endotracheal tube and develop into infection involving the respiratory airway, leading to ventilator-associated pneumonia. An estimated 300,000 ventilator-associated pneumonia occur in the United States annually, with a mortality rate of at least 33%.[16] Some data advocate the pre-operative use of antiseptic mouthwash to prevent or minimize the occurrence of pulmonary infections.[17]

Most mouthwashes contain either a high concentration of chlorhexidine (0.12%) or some alcohol. Mouthwashes, containing chlorhexidine or alcohol, were shown to promote a significant reduction in microbial load in the oral cavity.[18] Chlorhexidine is one of the most effective antimicrobial agents for controlling dental plaque, but is known to have a bitter taste. On the other hand, alcohol is a potent antimicrobial but is also irritating, especially to sensitive or inflamed mucosa. Both factors are of concern to the oral care of cancer patients, who are immunocompromised due to chemotherapy, and often suffer from mucositis, and hence would benefit from the use of a mild efficacious mouthwash. Unfortunately, there are few mouthwashes that are suitable for this group of patients, in terms of comfort and safety.

Chlorhexidine is a known antiseptic substance that is widely used in commercial mouthwash and other antiseptics. It is an efficacious antimicrobial agent for the control of dental plaque. Brilliant green is a triphenyl methane dye with an antiseptic activity when used in high concentration.

Brilliant green is used in high concentration, along with gentian violet and proflavine hemi sulfate, in the triple dye broad-spectrum antiseptic solution for neonatal nursery use.[19–22] The objective of the study was to develop an efficacious antimicrobial alcohol-free mouthwash that is non-staining, non-irritant, and that would be useful for the oral care of immunocompromised patients, such as patients with cancer, HIV patients, and those on steroids. We tested the solution against *Candida albicans*, a common cause of fungal infections in cancer patients, as well as against *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and MRSA, all being common sources of infection in immunocompromised patients. We also tested the efficacy of three other commercially available mouthwashes against these organisms.

Materials and methods

The model we used is based on the methods outlined in the federal registry for antiseptic drug products,[23] and which are commonly used to assess and compare antimicrobial activity of different oral antiseptic mouthwashes. The novel mouthwash tested, contained chlorhexidine and brilliant green, and is referred to in this manuscript as Gardine.

We tested the efficacy of Gardine against the free-floating form of *C. albicans*, *K. pneumoniae*, *P. aeruginosa*, and MRSA, and against the biofilm form of *C. albicans* (for adherence). We compared its efficacy with that of the following alcohol-based mouthwashes: PerioGard® (Colgate-Palmolive, Canton, MA), which contains 11.6% alcohol, Listerine® (Warner-Lambert, Morris Plains, NJ), which contains 26.9% alcohol, and Scope® (Proctor & Gamble, Cincinnati, OH), which contains 14.3% alcohol.

Preparation of the mouthwash solution

We used chlorhexidine in different low concentrations; 0.006% for testing the free-floating organisms, 0.012%, 0.024% for testing *C. albicans* biofilm, and 0.04% for the comparison with other mouthwashes. Chlorhexidine was combined with a low stainless concentration of 0.004 mg/mL of brilliant green dye. The mouthwash solution was prepared by mixing chlorhexidine gluconate solution with brilliant green powder that was previously dissolved in distilled water. We also used brilliant green at two concentrations of 0.004 mg/mL and 0.008 mg/mL.

Mouthwash activity against planktonic organism

After preparing the antiseptic solutions, inoculums of 1.5×10^8 colony forming units/mL (CFU/mL), made of a 0.5 McFarland standard, was prepared for the *C. albicans*, *K. pneumoniae*, *P. aeruginosa* and MRSA. A 500 µL of the prepared inoculum were then added to 500 µL of Gardine antiseptic solution and incubated at 37 °C for 10 min. A 100 µL were then pipetted and plated onto Trypticase soy agar (TSA) with 5% sheep blood plates and incubated at 37 °C overnight. Colonies were then counted up to 100 then multiplied by the dilution factor of 50 and documented.

Mouthwash activity against *Candida* in biofilm

We used a modification of a previously published bioprosthetic biofilm colonization model.[24] As a surface for biofilm adherence, we used discs, 20 mm in diameter and 2 mm in thickness, made of the same material as denture (mixture of polyacrylic powder and liquid acrylic monomer) that were manufactured at the dental laboratory of M.D. Anderson Cancer Center. We used artificial saliva without enzymes (to avoid any antibacterial effect) as a medium (Roxane Laboratories, Inc. Columbus, OH). Discs were soaked in saliva and were incubated overnight at 37 °C. Dental discs were removed from the saliva and placed into 50 mL tubes (6–7 pieces/tube) and covered with inoculum (~10 mL), equal to McFarland standard, and were incubated overnight at 37 °C in a shaker incubator. Inoculated broth was removed with pipettes; 10 mL of saline was added and incubated for 30 min, as a washing step, to get rid of any free-floating organisms. Discs were then removed gently and placed into separate clean tubes containing two Gardine solutions, prepared with two different chlorhexidine concentrations: 0.012% and 0.024%, as well as saline, as control, and were left to soak for 10 min in the shaker incubator at 37 °C. The antiseptic solution was then removed and the dental discs were transferred into fresh tubes, each containing 5 mL of 0.9% saline. The tubes were sonicated for 5 min, then vortexed for 30 s, to detach any remaining colonies from the surface of the discs. Then 100 µL were pipetted from each tube and spread on blood agar plates, and incubated overnight at 37 °C. For the control, the same steps were taken, using broth instead of the antiseptic solution. Colonies in each plate were counted up to 100, multiplied by the dilution factor 50, and findings were documented.

Comparison of Gardine's activity with other alcohol-containing mouthwashes

Gardine solution was prepared, using a higher concentration of chlorhexidine (0.04%) and 0.004 mg/mL of brilliant green. Equal amounts were obtained of three commercially available, alcohol-containing mouthwashes: Periogard®, Listerine®, and Scope®. Four inocula (1.5×10^8 CFU/mL) made of a 0.5 McFarland standard, were prepared of four microorganisms: *C. albicans*, *K. pneumoniae*, *P. aeruginosa*, and MRSA. A volume of 500 µL of each of the prepared inoculum were then added to 500 µL of each antiseptic solution and incubated at 37 °C for 10 min; 100 µL were then pipetted and plated onto TSA blood plates and incubated at 37 °C overnight. The number of colonies in the plate was counted up to 100 then multiplied by the dilution factor 50 and results were documented.

Results

Activity against free-floating organisms

Gardine, at its low concentration was active against the free-floating *C. albicans*, *K. pneumoniae*, and MRSA. Brilliant green alone, at a concentration of 0.004 mg/mL and 0.008 mg/mL was not active against all organisms, also chlorhexidine alone, at two low concentrations of 0.006% and 0.012%, produced a partial or no antimicrobial activity. However, Gardine solution inhibited the growth of the organisms, indicating a possible synergistic effect of brilliant green with the low concentration of chlorhexidine (Table 1).

Activity against *Candida* in biofilm

Gardine solution was active against the biofilm form of *C. albicans*. There was a complete eradication of *C. albicans* biofilm after being exposed to Gardine (0.008 mg/mL brilliant green and 0.012% chlorhexidine) and Gardine (0.008 mg/mL brilliant green and 0.024% chlorhexidine) solutions (Table 2).

Table 1 : Synergism of chlorhexidine and brilliant green in solutions towards three free-floating microorganisms

	Klebsiella pneumoniae CFU	MRSA CFU	*Candida albicans* CFU
Saline (control)	>5000	>5000	>5000
0.004 mg/mL brilliant green	>5000	>5000	>5000
0.008 mg/mL brilliant green	>5000	>5000	>5000
0.006% chlorhexidine	>5000	>5000	400
0.004 mg/mL brilliant green + 0.006% chlorhexidine	0	0	0
0.008 mg/mL brilliant green + 0.006% chlorhexidine	0	0	0
0.012% chlorhexidine	0	>5000	100
0.008 mg/mL brilliant green + 0.012% chlorhexidine	0	0	0

The last solution (0.008/0.012) was tested against MRSA only, expressed as CFU/disc.
CFU = colony forming units; MRSA = methicillin-resistant *Staphylococcus aureus*.

Table 2 Synergism of chlorhexidine and brilliant green in solutions against *Candida albicans* biofilm form, expressed as CFU/disk

Solution	*Candida albicans* CFU		
Saline (control)	>5000	>5000	>5000
0.008 Brilliant green	>5000	>5000	>5000
0.012 Chlorhexidine	>5000	>5000	>5000
0.024 Chlorhexidine	>5000	>5000	>5000
0.008 Brilliant green + 0.012 chlorhexidine	0, 0, 0		
0.008 Brilliant green + 0.024 chlorhexidine	0, 0, 0		

CFU = colony forming units; MRSA = methicillin-resistant *Staphylococcus aureus*.

Comparing Gardine activity with other alcohol-containing antiseptic mouthwashes

Gardine solution, prepared with 0.04% chlorhexidine, was active against free-floating forms of the four organisms tested, *C. albicans*, *P. aeruginosa*, *K. pneumoniae*, and MRSA. Gardine solution was as active as Periogard® and Listerine® mouth rinse solutions in completely eradicating the free-floating organisms, as shown on petri dishes by the absence of microorganisms' growth, while Scope® mouth rinse showed some growth with MRSA and *P. aeruginosa* (Table 3).

Discussion

This in vitro study showed that the experimental mouthwash, while being free of alcohol and containing reduced concentration of chlorhexidine (0.04%), was shown to be efficacious in inhibiting bacterial and candidal activity. Gardine, was as active as two of the three tested commercial mouthwashes, and more active than the third, against the free-floating *C. albicans*, *P. aeruginosa*, *K. pneumoniae*, and MRSA, which are common causes of nosocomial pneumonia and ventilator-associated pneumonia, while being alcohol-free and possibly with a less bitter taste than some available products, due to its low content of chlorhexidine. The solution also proved effective against *Candida* embedded in biofilm.

Investigators in a recent study,[25] found pre-intubation gargling with povidine–iodine solution to be effective in reducing the post-intubation nosocomial pneumonia, caused by general bacteria and MRSA colonization in the pharynx. In patients with cancer and those who are immunocompromised, however, the povidine–iodine solution could be very irritating. In another study, chlorhexidine gluconate 0.12% oral rinse was found to be useful in reducing nosocomial pneumonia in patients undergoing heart surgery.[26] The overall rate of nosocomial pneumonia was reduced by 52% in the chlorhexidine 0.12%-treated patients compared to Listerine® treated patients, while among patients intubated for more than 24 h, pneumonia rate was reduced by 58% ($P = 0.06$). Unfortunately, such level of chlorhexidine concentration (0.12%) could be irritating to critically ill and immunocompromised patients, who suffer from oral cavity ulcers and mucositis, in addition to its unpleasant taste. Ventilator-associated pneumonia is addressed in a CDC report, whereby it is emphasized that patients receiving mechanical ventilation are at highest risk for acquiring nosocomial pneumonia.[27] The use of antiseptic mouthwash, as part of a multifaceted protocol implemented in the intensive care unit, contributed to reducing the incidence of ventilator-associated pneumonia by about 50% ($P < 0.0001$).[28] In an in vivo study, oral colonizing bacteria increased significantly after rinsing with a sucrose solution. The oral bacteria were not affected by rinsing with water or fluoride mouth rinse, but were significantly inhibited by rinsing with chlorhexidine, cetylpyridinium chloride or triclosan/copolymer dentrifrice. A dose-dependent inhibition was noted with chlorhexidine rinses.[29] Chlorhexidine can directly damage the microbial cytoplasmic membrane as well as it can attach to a variety of substrates, allowing for the antimicrobial property of the chemical.[30]

Brecx et al. demonstrated, in a study of volunteers, that a combination of habitual self-performed and non-supervised oral hygiene with mouth rinse was found to be more beneficial for plaque control than the use of traditional oral hygiene alone. In addition, when mouth rinses were used to supplement routine oral hygiene, chlorhexidine was found to be the most powerful solution.[31]

However, while some authors report the potential benefit of chlorhexidine on oral mucositis and as an antimicrobial rinse, with antiplaque effects as well as antibacterial and antifungal activity, others report lack of impact against mucositis.[32,33] In a double blind randomized study of patients receiving radiation therapy of the oral cavity mucosa,

Table 3 Comparison between efficacy of alcohol-free Gardine solution and three alcohol-containing mouthwashes, against four microorganisms (free floating), expressed as CFU/disk

Solution	MRSA	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*	*Candida albicans*
Saline (control)	>5000, >5000, >5000	>5000, >5000, >5000	>5000, >5000, >5000	>5000, >5000, >5000
0.004 Brilliant green + 0.04% chlorhexidine	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
Periogard®	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
Listerine®	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
Scope®	50, 0, 0	1000, 0, 0	0, 0, 0	0, 0, 0

CFU = colony forming units; MRSA = methicillin-resistant *Staphylococcus aureus*.

chlorhexidine mouthwash was found to be associated with mouthwash-induced discomfort, taste alteration, and teeth staining, more than the placebo mouthwash.[33]

On the other hand, chlorhexidine was found to be beneficial in controlling some carcinogenic substances. A study concluded that chlorhexidine mouthwash significantly reduced salivary acetaldehyde production, a metabolite resulting from ethanol consumption and which has been shown to have multiple mutagenic effects and to be carcinogenic to animals.[34]

The three commercially available mouthwash solutions that were tested in this study contain alcohol in variable concentrations; PerioGard® has 11.6% alcohol, Listerine® has 26.9% alcohol, and Scope® has 14.3% alcohol. It should be noted that alcohol in mouthwash is contraindicated in certain high-risk patients, including those who have mucositis, those undergoing head and neck irradiation, those who are alcoholic, and those who are immunocompromised.[35,36] Winn et al. found an association between the risk of oral cancer and the frequent and prolonged use of alcohol-based mouthwashes with high alcohol contents. The risk of oral cancer increased by 40–60%, after adjusting for other risk factors, such as tobacco and alcohol consumption.[37] Hence it is preferable, if possible to avoid the use of mouthwash with high alcohol content. Eldridge et al. addressed earlier the antimicrobial efficacy of an alcohol-free chlorhexidine mouth rinse,[38] and found no difference between the commercial alcohol-based chlorhexidine 0.12% and the alcohol-free chlorhexidine 0.12% through both in vitro and in vivo studies. The Gardine solution could prove to be advantageous in that it is alcohol-free and contains lower chlorhexidine concentration.

Gram-negative bacteria, such as *K. pneumoniae* and *P. aeruginosa*, and Gram-positive bacteria, such as MRSA that frequently colonize the oral cavity of hospitalized patients,[39,40] have emerged as causes of nosocomial pneumonia. This has stimulated the search for preventive and therapeutic measures to minimize oral and respiratory colonization by the simple use of a broad- spectrum antiseptic mouthwash pre-operatively or pre-intubation.[26,28] In this study, Gardine solution proved effective against the free-floating forms of these bacteria.

In addition to mouth and respiratory tract colonization with multidrug-resistant bacteria, *Candida* infections have shown a substantial increase in the United States during the last two decades. This has contributed to the rise in prolonged hospitalization and related deaths. *Candida* species have been shown to be the fourth most common group of organisms causing nosocomial bloodstream infections in the United States[41] Factors contributing to this trend include a growing population of immunocompromised patients, such as HIV, cancer, chronic use of steroids, and transplant patients, as well as the use of new aggressive and invasive therapeutic strategies such as irradiation.

Among immunocompromised patients and patients with cancer, candidiasis is more prevalent in patients with hematologic malignancies than in those with solid tumors and among neutropenic patients than otherwise.[42] The experimental antiseptic mouthwash, Gardine, provided coverage against *C. albicans*, and it may prove suitable for use in immunocompromised patients, providing a valuable protection against this opportunistic nosocomial infection.

This current study has several limitations, including those inherent to its in vitro design, such as the limitations of extrapolating from this in vitro study to intact mammalian systems, and hence the need for in vivo studies. While the study addressed the effectiveness of Gardine solution in preventing the free-floating forms of bacteria and fungi, as well as fungal biofilm formation, biofilm testing for bacteria needs to also be studied. Additional in vitro as well as in vivo studies should be performed to further modify the alcohol-free mouthwash solution so that it may provide a potent coverage to broader spectrum of the organisms that colonize the oral cavity. Although neither chlorhexidine alone nor brilliant green alone is cytotoxic, testing Gardine for cytotoxicity is important if it is to be considered for further use.

In conclusion, Gardine solution showed an antimicrobial activity against *C. albicans* and other common bacteria. Its efficacy against the biofilm-embedded *C. albicans* has also been shown. The antiseptic activity of Gardine demonstrated a comparable activity with other commercially available, alcohol-containing mouthwashes. The novel Gardine solution may serve as a convenient alternative mouthwash for immunocompromised cancer patients and for preoperative patients at high risk for nosocomial pneumonia. Its low concentration of chlorhexidine may minimize the unpleasant taste, thus enhancing patient compliance. Furthermore, being alcohol-free makes it non-irritant, thus gentler to use for patients with sensitive or inflamed mucosa.

References

1. Abu-Elteen KH, Abu-Alteen RM. The prevalence of *Candida albicans* populations in the mouths of complete denture wearers. *New Microb* 1998;21(1):41–8.
2. Budtz-Jorgensen E, Stenderup A, Grabowski M. An epidemiological study of yeasts in elderly denture wearers. *Community Dent Oral Epidemiol* 1975;3:115–9.
3. Webb BC, Thomas CJ, Willcox MD, Harty DW, Knox KW. *Candida*-associated denture stomatitis. Aetiology and management: a review. Part 1. Factors influencing distribution of *Candida* species in the oral cavity. *Aust Dent J* 1998;43:45–50.
4. Belazi M, Velegraki A, Koussidou-Eremondi T, Andreadis D, Hini G, Arsenis G, et al. Oral *Candida* isolates in patients undergoing radiotherapy for head and neck cancer: prevalence, azole susceptibility profiles and response to antifungal treatment. *Oral Microb Immunol* 2004;19:347–51.
5. Epstein JB, Silverman Jr S, Paggiarino DA, Crockett S, Schubert NN, Senzer NN, et al. Benzydamine HCl for prophylaxis of radiation-induced oral mucositis: results from a multicenter, randomized, double-blind, placebo-controlled clinical trial. *Cancer* 2001;92:875–85.
6. Webb BC, Thomas CJ, Whittle T. A 2-year study of *Candida*-associated denture stomatitis treatment in aged care subjects. *Gerodontology* 2005;22(3):168–76.
7. Epstein JB, Hancock PJ, Nantel S. Oral Candidiasis in hematopoietic cell transplantation patients: an outcome-based analysis. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2003;96:154–63.
8. Odds FC. *Candida and candidiasis. A review and bibliography*. 2nd ed. London: Bailliere Tindall; 1988, p. 117.
9. Stevens DA, Greene SI, Lang OS. Thrush can be prevented in patients with acquired immunodeficiency syndrome and the acquired immunodeficiency syndrome-related complex.

Randomized double blind, placebo-controlled study of 100 mg oral fluconazole daily. *Arch Intern Med* 1991;51:2458–64.
10. Tapper-Jones LM, Aldred MJ, Walker DM, Hayes TM. Candidal infections and populations of *Candida albicans* in mouths of diabetics. *J Clin Pathol* 1981;34:706–11.
11. Bodey G, Bueltmann B, Duguid W, Gibbs D, Hanak H, Hotchi M, et al. Fungal infections in cancer patients: an international autopsy survey. *Eur J Clin Microb Infect Dis* 1992;11(2): 99–109.
12. Lifson AR, Hilton JF, Westenhouse JL, Canchola AJ, Samuel MC, Katz MH, et al. Time from HIV seroconversion to oral candidiasis or hairy leukoplakia among homosexual and bisexual men enrolled in three prospective cohorts. *AIDS* 1994;8(1):73–9.
13. Worthington HV, Clarkson JE. Prevention of oral mucositis and oral candidiasis for patients with cancer treated with chemotherapy. *Cochrane Syst Rev* 2002;66(8):903–11.
14. Woo SB, Sonis ST, Monopoli MM, Sonis AL. A longitudinal study of oral ulcerative mucositis in bone marrow transplant recipients. *Cancer* 1993;72(5):1612–7.
15. Cayton RM, Soutar CA, Stanford CF, Turner GC, Nunn AJ. Double-blind trial comparing two dosage schedules of beclomethasone dipropionate aerosol in the treatment of chronic bronchial asthma. *Lancet*(August):303–7.
16. Safdar N, Crnich CJ, Maki DG. The pathogenesis of ventilator-associated pneumonia: its relevance to developing effective strategies for prevention. *Respir Care* 2005;50(6):725–41.
17. Okuda M, Kaneko Y, Ichinohe T, Ishihara K, Okuda K. Reduction of potential respiratory pathogens by oral hygienic treatment in patients undergoing endotracheal anesthesia. *J Anesth* 2003;17(2):84–91.
18. Shapiro S, Giertsen E, Guggenheim B. An in vitro oral biofilm model for comparing the efficacy of antimicrobial mouth rinses. *Caries Res* 2002;36(2):93–100.
19. Green FJ. *The sigma–Aldrich handbook of stains, dyes and indicators*. Milwaukee, WI: Aldrich Chemical, Co., Inc.; 1990, p 160.
20. Remington JP. *Remington's pharmaceutical sciences*. 13th ed. Mack: Easton, PA; 1965, p 1257.
21. Rogers TH. Inhibition of sulfate-reducing bacteria in dyes. *J Soc Chem Ind* 1940;59:34–9.
22. Tests and standards. New and unofficial remedies, American Medical Association. Lippincott: Philadelphia; 1953. p. 4.
23. Tentative final monograph for antiseptic drug products. *Federal Register* 1994;591994., Sec. 356.90.
24. Kuhn DM, George T, Chandra J, Mukherjee PK, Ghannoum MA. Antifungal susceptibility of *Candida* biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. *Antimicrob Agents Chemother* 2002;46:1773–80.
25. Ogata J, Minami K, Miyamoto H, et al. Gargling with povidone–iodine reduces the transport of bacteria during oral intubation. *Can J Anaesth* 2004;51(9):932–6.
26. Houston S, Hougland P, Anderson JJ, LaRocco M, Kennedy V, Gentry LO. Effectiveness of 0.12% chlorhexidine gluconate oral rinse in reducing prevalence of nosocomial pneumonia in patients undergoing heart surgery. *Am J Crit Care* 2002;11(6):567–70.
27. Centers for Disease Control and Prevention Guidelines for Prevention of Nosocomial Pneumonia. *MMWR* 1997;46 (RR-1):79.
28. Baxter AD, Allan J, Bedard J, et al. Adherence to simple and effective measures reduces the incidence of ventilator-associated pneumonia. *Can J Anaesth* 2005;52(5):535–41.
29. Jenkins S, Addy M, Newcombe RG. Dose response of chlorhexidine against plaque and comparison with triclosan. *J Clin Periodontol* 1994;21:250–5.
30. Stanley A, Wilson M, Newman HN. The in vitro effects of chlorhexidine on subgingival plaque bacteria. *J Clin Periodontol* 1989;16:259–64.
31. Brecx M, Brownstone E, Macdonald L, Gelskey S, Cheang M. Efficacy of Listerine, Meridol and chlorhexidine mouthrinses as supplements to regular tooth cleaning measures. *J Clin Periodontol* 1992;19(3):202–7.
32. Hancock PJ, Epstein JB, Sadler GR. Oral and dental management related to radiation therapy for head and neck cancer. *J Can Dent Assoc* 2003;69:585–90.
33. Foote RL, Loprinzi CL, Frank AR, O'Fallon JR, Gulavita S, Twefik HH, et al. Randomized trial of a chlorhexidine mouthwash for alleviation of radiation-induced mucositis. *J Clin Oncol* 1994;12(12):2630–3.
34. Homann N, Jousimies-Somer H, Jokelainen K, Heine R, Salaspuro M. High acetaldehyde levels in saliva after ethanol consumption: methodological aspects and pathogenetic implications. *Carcinogenesis* 1997;18(9):1739–43.
35. Sreenivasan PK, Mattai J, Nabi N, Xu T, Gaffar A. A simple approach to examine early oral microbial biofilm formation and the effects of treatments. *Oral Microb Immunol* 2004;19(5):297–302.
36. Elmore JG, Horwitz RI. Oral cancer and mouthwash use: evaluation of the epidemiologic evidence. *Otolaryngol Head Neck Surg* 1995;113(3):253–61.
37. Winn DM, Blot WJ, McLaughlin JK, et al. Mouthwash use and oral conditions in the risk of oral and pharyngeal cancer. *Cancer Res* 1991;51:3044–7.
38. Eldridge KR, Finnie SF, Stephens JA, Mauad AM, Munoz CA, Kettering JD. Efficacy of an alcohol-free chlorhexidine mouthrinse as an antimicrobial agent. *J Prosthet Dent* 1998;80(6): 685–90.
39. Dantas SR, Moretti-Branchini ML. Impact of antibiotic-resistant pathogens colonizing the respiratory secretions of patients in an extended-care area of the emergency department. *Infect Control Hosp Epidemiol* 2003;24(5):351–5.
40. Martins ST, Moreira M, Furtado GH, et al. Application of control measures for infections caused by multi-resistant gram-negative bacteria in intensive care unit patient. *Mem Inst Oswaldo Cruz* 2004;99(3):331–4.
41. Edmond MB, Wallace RP. Nosocomial bloodstream infections in United States hospitals: a three-year analysis. *Clin Infect Dis* 1999;29:239–44.
42. Myoken Y, Sugat T, Fujita Y, Kohara T, Mikami Y. Oropharyngeal *Candida* colonization and infection in neutropenic patients with hematologic malignancies. *Oral Surg Oral Med Oral Med Oral Radiol Endod* 2004;97(2):137–8.